REPRINTED FROM FOOD TECHNOLOGY IN AUSTRALIA
VOLUME 36 NO. 1, JANUARY 1984 PP 46-48

The use of high performance liquid chromatography for the analysis of ascorbic acid and dehydroascorbic acid in orange juice and powdered orange drink

P.R. HADDAD and J. LAU

Ascorbic acid and dehydroascorbic acid may be separated by high performance liquid chromatography (HPLC) after pre-column reaction of dehydroascorbic acid with 1,2-phenylenediamine. Two methods are described, viz. reversed-phase ion-pairing HPLC, and the use of a CN column for the separation. When both methods were applied to the analysis of orange juice and powdered orange drink, the reversed-phase ion-pairing method was found to be subject to interference problems and variability in retention times. The CN column method was more successful, particularly for powdered orange drink. Recovery and precision data are presented.

The determination of ascorbic acid (AA) in foodstuffs has been an important area of analytical method development for many years. Many analytical procedures have been reported, including redox titrimetric, spectro-photometric, fluorometric and electrochemical techniques. However, the principal problem encountered with these methods is not one of sensitivity required for routine analysis of foodstuffs, but rather the interfering species present in the samples. This situation has led to increased research into the application of chromatographic methods of analysis, particularly high performance liquid chromatography (HPLC), where the ascorbic acid is separated from interfering species before its determination. Various column types and mobile phases have been studied. When columns containing a bonded hydrocarbon functionality, such as C_2, C_8 or C_{18}, were used, adequate retention of the AA was achieved only with the use of hydrophobic pairing ions such as tetrabutylammonium phosphate (Finley & Duang 1981, Wills *et al.* 1977), tridecylammonium formate (Sood *et al.* 1976) or hexadecyltrimethylammonium bromide (Vandermark 1980) in the mobile phase. AA has also been successfully determined using ion-exchange (Pachla & Kissinger 1976, Williams, Baker & Schmit 1973, Rouseff 1979) and bonded amino-functionality columns (Bui-Nguyen 1980). However, it has been reported that ion-exchange columns easily became irreversibly poisoned (Singh 1974), and that the mobile phases used with the amino column seriously reduced the lifetime of the column (Carnevale 1980). The use of a μ-Bondapak/Carbohydrate column for the separation of ascorbic acid and dehydroascorbic acid has also been recently reported (Wimalasiri & Wills 1983), where the dehydroascorbic acid was detected at 214 nm.

Dehydroascorbic acid (DAA) is produced when ascorbic acid is oxidised by mild oxidising agents and has similar antiscorbutic activity to AA; its determination is therefore important. Indeed, the 'total vitamin C' content of a sample comprises the sum of the AA and DAA acid contents. HPLC is ideally suited to the separation of AA and DAA, but the latter component is somewhat difficult to detect because of its low UV absorptivity, even at wavelengths in the range 210–215 nm.

An accepted instrumental method for the determination of AA involves its oxidation to DAA, followed by reaction with 1,2-phenylenediamine to produce the fluorophor 3-(1,2-dihydroxyethyl) furo [3,4-b] quinoxaline-1-one (DFQ), the amount of which is determined by spectrofluorometry (Deutsch & Weeks 1965). This method has recently been adapted in this laboratory to permit the simultaneous detemination of AA and DAA by reacting the latter with 1,2-phenylenediamine and separating the resultant DFQ and AA using reversed-phase ion-pair HPLC with UV detection (Keating & Haddad 1982).

This paper reports the results obtained when the above separation procedure was applied; it also reports a modified approach using a cyano-bonded phase column to the simultaneous determination of AA and DAA in commercial orange juice and powdered orange drink.

Experimental

Instrumentation and reagents

The liquid chromatograph used consisted of a Waters Assoc. (Milford, MA, USA) Model M6000 pump, Model U6K injector, Model M450 variable-wavelength detector and an Omniscribe Model B5217-1 recorder. Waters Assoc. μ-Bondapak CN and μ-Bondapak C18 columns were used. Analytical grade methanol was doubly distilled from all glass apparatus and water was distilled using a Millipore Milli-Q water purification system. Solvents were filtered and degassed before use.

The following reagents were used: L(+)-ascorbic acid (Merck, Darmstadt, G.F.R.), dehydroascorbic acid (Pfaltz and Bauer, Stamford, CT.), 1,2-phenylenediaminedihydrochloride and hexadecyltrimethylammonium bromide (both from Fluka, Buchs, Switzerland).

Sample preparation

A stock solution was prepared by dissolving DAA (approx.

Dr Paul Haddad is a lecturer and Joseph Lau is a postgraduate student in the Department of Analytical Chemistry, The University of New South Wales, PO Box 1, Kensington, NSW 2033.

0015-6647/84/36/0046/03e 1984

9 mg) and AA (45 mg) in water (50 mL). Simultaneous calibration plots were constructed for DAA and AA by adding suitable aliquots (0–8 mL) of the above stock solution to a series of 25 mL volumetric flasks containing 2 mL of an aqueous solution of 1,2-phenylenediaminedihydrochloride (3.6 g/100 ml) and diluting to the mark with extracting solution (7.5 g metaphosphoric acid in 250 mL 1M acetic acid solution). These solutions were held in a waterbath at 40°C for 90 min, and an aliquot of each was then chromatographed using the procedure described below.

A commercial orange juice sample and a powdered orange drink product were analysed as follows: 15 mL of either orange juice or orange drink, prepared according to the manufacturer's instructions, were added to a 25 mL volumetric flask and an identical procedure to that described above for the calibration data was followed. Standard addition techniques were also employed. All samples were filtered before chromatographic analysis.

Chromatographic procedure

When the C_{18}-column was used for analysis, the mobile phase consisted of methanol/water (60/40 v/v) containing 2.5 x 10^{-4} M hexadecyltrimethylammonium bromide. The detector wavelength was initially set at 348 nm until DFQ had eluted, after which it was changed to 290 nm for the detection of ascorbic acid. The detector sensitivity was maintained at 0.1 AUFS. For the CN-column, the mobile phase used was acetic acid (20 g/L)–methanol (95:5). The detector was initially operated at 254 nm and 1.0 AUFS until the ascorbic acid had eluted, after which it was altered to 348 nm and 0.1 AUFS for the detection of DFQ.

Results and discussion

Optimum reaction conditions

The instability of AA, DAA and DFQ are well recognised (Deutsch & Weeks 1965). It is customary to use a stabilising solution such as metaphosphoric acid-acetic acid for extraction of DAA and AA from samples. The formation of DFQ from DAA and 1,2-phenylenediamine is very time dependent; DFQ is also sensitive to light.

The ability chromatographically to separate DFQ from the reaction mixture (using the HPLC approaches described in this paper) provided a suitable method for the determination of optimum reaction time. It was observed that the rate of formation of DFQ in aqueous solution was extremely slow, with up to 30 h reaction time being required for the DFQ peak area to reach a stable value. The same result was observed when metaphosphoric acid-acetic acid solution was used for the reaction medium. The results differ greatly from those obtained by Deutsch and Weeks (1965), who found that the fluorescence of DFQ reached a maximum value within 40 min of commencement of the reaction between dehyroascorbic acid and 1,2-phenylenediamine. It is possible that fluorescence quenching effects could cause a different apparent reaction time when the fluorescence monitoring method was used; however, this would not explain such a large discrepancy in the results obtained by the two methods.

In a previous paper (Keating & Haddad 1982) it had been reported that both the rate of formation and stability of DFQ were considerably enhanced if methanol was added to the reacting solution. For the analysis of orange juice, however, addition of methanol is undesirable because of the resultant dilution of the sample; we have elected to use metaphoric acid-acetic acid solution as the reaction medium, since AA and DAA extracts from solid samples, such as dried foods, are commonly prepared in this solvent. Clearly, a more rapid rate of formation of DFQ in this solvent is desirable and further studies have shown that use of a reaction temperature of 40°C produced stable DFQ peak areas after 90 min, with the DFQ remaining stable for at least a further 3 h. For this reason, all reactions were carried out at 40°C.

Reversed-phase ion-pairing method

A typical separation of AA and DFQ, obtained using pure reagents, is shown in Figure 1. The wavelength was initially set at 348 nm for the detection of DFQ, after which it was manually

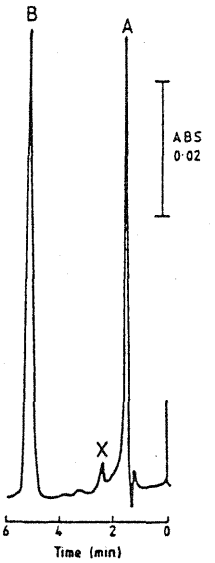

Figure 1. Separation of DFQ (A) and ascorbic acid (B) using the reversed-phase ion-paring method

Conditions: mobile phase, methanol–water (60:40) containing 2.5 x 10^{-4}M hexadecyltrimethylammonium bromide; flow rate 2.0 mL/min; detector sensitivity, 0.1 AUFS; wavelength, 348 nm initially and changed to 290 nm at point x; sample, 10 µL injection of a solution containing 25 mg ascorbic acid/100 mL and 5 mg dehydroascorbic acid/100 mL, derivatised with 1,2-phenylenediamine

Reproduced with permission from Keating and Haddad (1982)

changed to 290 nm for AA detection. The latter wavelength is relatively insenitive for AA but was deliberately selected so that the same detector sensitivity could be used for the typical concentrations of AA and DAA found in fruit juices.

Linear calibration plots were obtained for DAA in the range 0–6 mg/100 mL and for AA in the range 0–30 mg/100 mL although it is probable that linearity extended beyond these concentration ranges. The precision obtained for six replicate standard solutions containing 5 mg/100 mL of DAA and 30 mg/100 mL of AA in the final solution was 1.5% RSD and 3.9% RSD for the AA and DFQ peak heights, respectively. When the same method was applied to the analysis of orange juice and powdered orange drink, very poor results were obtained. Recoveries of added DAA averaged 142.3% and 125.2% for the orange juice and powdered orange drink, respectively. The equivalent results for average recovery of added AA were 81.3% and 91.5%. The amounts of AA and DAA used for these recovery studies were identical to those listed in Table 1 for the CN column method. The precision values obtained for both types of samples were 12.3% RSD for DAA and 8.6% RSD for AA. These results are in strong contrast to those obtained when the method was tested on standard mixtures of the components (see above), and clearly indicate the unsuitability of this approach for the samples studied.

Two major problems were encountered in the analysis of these samples; firstly, the DFQ eluted very close to the void volume and the interferences resulting from unretained components of the samples were largely responsible for the poor results for DAA described earlier. Secondly, the retention time for AA varied considerably (by up to 40%) from sample to sample. This is understandable in terms of the low concentration of ion pairing reagent used (2.5 x 10^{-4}M), together with the high ionic content of the samples studied. Competition between AA and other ionic species for the ion-pairing reagents is likely to cause significant changes in retention. Incomplete equilibration of the column with the ion pairing reagent is a further possible explanation for the observed variation in retention time.

CN Column method

In view of the deficiencies of the reversed-phase ion-pairing

Table 1. Recovery data for dehydroascorbic and ascorbic acids using the CN column method

Sample	Dehydroascorbic acid			Ascorbic acid		
	Amount added (mg/100 mL)	Amount found (mg/100 mL)	Recovery (%)	Amount added (mg/100 mL)	Amount found (mg/100 mL)	Recovery (%)
Powdered orange drink	0	1.91	—	0	13.71	—
	2.35	4.13	95	12.75	26.53	101
	4.70	6.84	105	25.50	38.92	99
Commercial orange juice	0	0.51	—	0	13.75	—
	2.35	3.49	127	12.75	25.57	93
	4.70	5.09	98	25.50	38.80	98

Each result shown is the average of four determinations

method, an alternative approach was sought. It has recently been reported (Carnevale 1980) that ascorbic acid may be separated from benzoic and sorbic acids on a CN column without the use of ion-pairing reagents. All three of the above components could be determined in a single chromatographic run if appropriate changes of wavelength and detector sensitivity were employed. The success of this method suggested the examination of the same conditions for the separation of AA and DFQ.

Figure 2 shows a chromatogram obtained using a standard mixture of AA and DAA. AA eluted first and showed sufficient retention to be clearly removed from the void volume peak, and the DFQ was also well resolved. In an effort to optimise the results, a sensitive wavelength (254 nm) was employed for AA; this necesitated a change of detector sensitivity as well as wavelength for the detection of DFQ. Once again, linear calibration plots were obtained over the ranges 0–6 mg/100 mL for DAA and 0–30 mg/100 mL for AA, expressed as the concentration of solute in the final injected solution. Results of recovery studies performed on orange juice and powdered orange drink samples are given in Table 1.

Precision values obtained for five replicate determinations of DAA (5 mg/100 mL) were 5.9% RSD and 1.6% RSD for the fruit juice and powdered orange drink, respectively, whilst the equivalent results for AA (38 mg/100 mL) were 3.2% RSD and 2.9% RSD, respectively.

These results suggest that the determination of DAA using the CN column method is applicable only to the analysis of the powdered orange drink sample which as expected, contained a relatively high level of DAA. The variable recovery and poorer precision obtained with the orange juice suggest that interference problems still exist with this type of sample. Attempts to identify and remove these co-eluting interfering species were not successful and adjustment of the mobile phase composition failed to provide resolution of DFQ from the interferences. The outcome of this drawback is that the CN column method cannot be used with acceptable confidence for the analysis of DAA in orange juice samples. On the other hand, the results obtained for AA with both types of sample were quite acceptable, despite the fact that the particular samples analysed contained relatively small amounts of AA. More concentrated samples may be readily analysed by taking a smaller aliquot of sample than that used for the particuar samples described above.

Conclusions

The reversed-phase ion-pairing method for the separation of AA and DFQ is not applicable to the analysis of orange juice or powdered orange drink samples due to interference problems and variable retention times. An alternative method using a CN column without an ion pairing reagent has been evaluated; it was found that this approach eliminated the problem of variable retention times for AA. On the other hand, the problem of coeluting interferences could not be overcome in the CN column method when orange juice samples were analysed; however, the method was found to be suitable for the analysis of AA and DAA in the less complex and sample matrix of powdered orange juice drink.

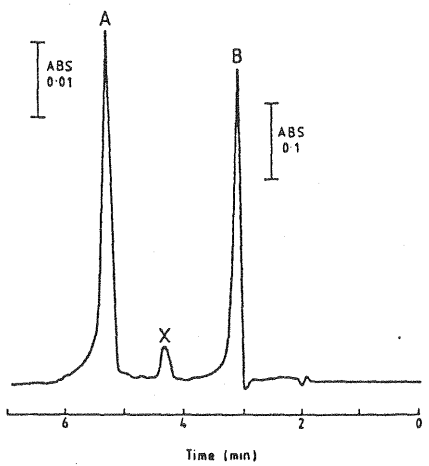

Figure 2. Separation of DFQ (A) and ascorbic acid (B) using the CN column method

Conditions: mobile phase, 2% acetic acid–methanol (95:5); flow rate 1.0 mL/min; detector set at 254 nm and 1.0 AUFS initially and changed at point x to 348 nm and 0.1 AUFS; sample, 10 µL injection of a solution containing 21.6 mg ascorbic acid/100 mL and 4.3 mg dehydroascorbic acid/100 mL, derivatised with 1,2-phenylenediamine

References

Bui-Nguyen, M.H. Application of high performance liquid chromatography to the separation of ascorbic acid from isoascorbic acid. J. Chromatogr. 196: 163–5; 1980.

Carnevale, J. Determination of ascorbic acid and benzoic acids in citrus juices by high-performance liquid chromatography. Food Technol. Aust. 32: 302–5; 1980.

Deutsch, M.J. & Weeks, C.E. Microfluorometric assay for vitamin C. J. Ass. Offic. Anal. Chem. 48: 1248–56; 1965.

Finley, J.W. & Duang, E. Resolution of ascorbic, dehydroascorbic and diketogulonic acids by paired-ion reversed-phase chromatography. J. Chromatogr. 207: 449–53; 1981.

Keating, R.W. & Haddad, P.R. Simultaneous determination of ascorbic acid and dehydroascorbic acid by reversed-phase ion-pair high performance liquid chromatography with pre-column derivatisation. J. Chromatogr. 245: 249–55; 1982.

Pachla, L.A. & Kissinger, P.T. Determination of ascorbic acid in foodstuffs, pharmaceuticals and body fluids by liquid chromatography with electrochemical detection. Anal. Chem. 48: 364–7; 1976.

Rouseff, R. Liquid chromatographic analysis of food and beverages. Vol. I. Charalambous, G., ed. New York: Academic Press; 1979: 161.

Singh, M. Determination of uncombined intermediates in FD & C yellow No. 6 by high pressure liquid chromatography. J. Ass. Offic. Anal. Chem. 57: 358–9; 1974.

Sood, S.P., Sartori, L.E., Wittmer, D.P. & Haney, W.G. High-pressure liquid chromatographic determination of ascorbic acid in selected foods and multivitamin products. Anal. Chem. 48: 796–8; 1976.

Vandermark, F.C. A liquid chromatography procedure for determining water-soluble vitamins in pharmaceutical preparations. Chromatogr. Newsl. 8: 27–8; 1980.

Williams, R.C., Baker, D.R. & Schmit, J.A. Analysis of water-soluble vittamins by high-speed ion-exchange chromatography. J. Chromatogr. Sci. 11: 618–24; 1973.

Wills, R.B.H., Shaw, C.G. & Day, W.R. Analysis of water soluble vitamins by high pressure liquid chromatography. J. Chromatogr. Sci. 15: 262–6; 1977.

Wimalasiri, P. & Wills, R.B.H. Simultaneous analysis of ascorbic acid and dehydroascorbic acid in fruit and vegetables by high performance liquid chromatography. J. Chromatogr. 256: 368–71; 1983.